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The first evidence of lumiphorbol as a metabolite of the *Euphorbia* species

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Abstract: *Euphorbia nicaeensis* All. belongs to the flowering plant family Euphorbiaceae. Our previous research has shown that the latex of this species is a rich source of jatrophone-type diterpenes. Due to the exceptional biological activities exhibited by these compounds, the research was extended to the root of this plant species. From the roots of *E. nicaeensis*, collected in Deliblato Sand (Serbia), a diterpene from an extremely rare class of lumiphorbol was isolated for the first time from *Euphorbia* species. The structure of the isolated compound was determined by spectroscopic techniques (1D- and 2D-NMR), as well as HRESIMS data.

Keywords: *Euphorbia nicaeensis*; Euphorbiaceae; diterpenes; lumiphorbol.

INTRODUCTION

Species of the *Euphorbia* genus are well known for their impressive structural diversity and adaptability to different environments.¹ These plants produce a wide range of secondary metabolites, among which diterpenes stand out due to their bioactivity and potential application in modern medicine.² Diterpenes from *Euphorbia* species are structurally diverse, including compounds such as phorbol esters, ingenane derivatives, jatrophone diterpenoids and many others.^{3–5} These metabolites often have protective roles in plants, such as deterring herbivores and protecting themselves from pathogens.^{5–8} However, their importance goes beyond the approved protective role in plants, as studies have shown promising pharmacological properties of these metabolites. A well-known group of diterpenes derived from *Euphorbia* are phorbol esters. These compounds are known for their

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ability to activate protein kinase C (PKC), an enzyme involved in cellular signaling pathways that regulate various cellular functions, including proliferation, differentiation and apoptosis.⁹ Although phorbol esters are associated with tumour-promoting activity, their mechanism of action has been crucial in cancer research, helping to develop therapeutic strategies targeting PKC.⁹ On the other hand, many phorbol derivatives have been shown not to have tumour-promoting effects, but rather exhibit anticancer activity against certain human cancer cell lines.^{10–12} Additionally, tiglane derivatives have demonstrated strong inhibitory activity against HIV-1 and HIV-2.¹³ Ingenane derivatives play a significant role in modern medicine. Ingenol-mebutate, derived from *Euphorbia peplus*, was approved for the topical treatment of actinic keratosis, a precursor of skin cancer.¹⁴ Its mechanism involves inducing rapid cell death in abnormal cells while minimizing damage to healthy tissue.¹⁴ However, it was withdrawn from use in the European Union in 2019 due to safety concerns.¹⁵ Research indicated a potential link between long-term use of ingenol-mebutate and an increased risk of skin cancer, including basal cell carcinoma and squamous cell carcinoma, and that the drug's risks outweigh its benefits.¹⁵ The most specific and rare class of diterpenes isolated from the *Euphorbia* genus are lumiphorbols, which are considered phorbol derivatives. It has been experimentally proven that lumiphorbols are derived from tiglane esters, where rings A and B are *cis* connected. The [2+2] cycloaddition, involving the double bonds at positions C1(2) and C6(7), occurs in the presence of light with a wavelength of 254 nm under laboratory conditions, while the mechanism of their formation in plants, particularly in the root, which is not exposed to UV radiation, remains unknown. A notable example is lumiphorbol triacetate, a cage derivative of 4 α -phorbol, which was first described by E. Hecker and his colleagues in 1968.¹⁶ In their study, Hecker *et al.* detailed the synthesis of lumiphorbol triacetate *via* ultraviolet (UV) irradiation of 4 α -phorbol-12,13,20-triacetate. This process induces an intramolecular cycloaddition, resulting in the unique cage-like structure of lumiphorbol.¹⁷ Due to its extremely specific structure, the biological activity of lumiphorbol derivatives was also investigated. For example, the ability of lumiphorbol-12,13,20-triacetate to activate PKC was evaluated. This compound was found to activate PKC by 93 % at high concentrations, while no activation was observed at standard concentration.¹⁸

Our previous study,¹⁹ where the subject of investigation was the latex of *E. nicaeensis*, showed that the species is a rich source of jatrophone derivatives that showed a strong inhibition of P-gp, so after the initial investigation, the research was extended to the root of the species.

EXPERIMENTAL

Apparatus and reagents

The optical rotations were measured on an Autopol IV (Rudolph Research Analytical) polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. All NMR data were

acquired on Bruker Avance III 500 NMR spectrometer (500 MHz for ^1H - and 125 MHz for ^{13}C -NMR, in CDCl_3 , with TMS as internal standard). The NMR spectra were analysed using TopSpin 3.6.2 software. The high-resolution LC/ESI positive TOF mass spectra were measured on a HPLC instrument (Agilent 1200 Series) coupled with a 6210 Time-of-Flight LC/MS system (Agilent Technologies). NP-HPLC-DAD: Agilent Technologies 1260 Series liquid chromatograph equipped with diode-array detector, autosampler, and collector; Zorbax RX-Sil (250 mm $\times$ 9.4 mm; 5 μm) column. RP-HPLC-DAD: Agilent Technologies 1100 Series liquid chromatograph equipped with diode-array detector, autosampler, and collector; Zorbax XDB-C18 column (250 mm $\times$ 9.4 mm; 5 μm). Dry-column flash chromatography (DCFC, Shusterman *et al.*, 1997) was performed on silica gel (ICN Silica 12–26 60 Å, Merck). Silica gel 60 F254 precoated aluminium sheets (0.25 mm, Merck) for TLC control were used. The TLC plates were visualized under a UV lamp at 254 nm and detected by spraying with solution of cerium molybdate in sulphuric acid, followed by heating. All solvents used for HPLC were HPLC grade, while all used solvents for DFCC and TLC were at least of analytical grade.

Plant material

The roots of *Euphorbia nicaeensis* were collected at Deliblato Sands (Serbia), collection site at latitude: 44°56'57" N and longitude: 21°11'13" E, in May 2018. The plant was identified by Professor Petar Marin, University of Belgrade – Faculty of Biology, Institute of Botany. Voucher specimen (No. 16855) has been deposited at the Herbarium of Botanical Garden “Jevremovac”, University of Belgrade, Belgrade (Serbia).

Isolation and purification

The process of isolating lumiphorbol involved the preparation of a root extract through continuous extraction with a 96 % aqueous ethanol solution. 152 g of ground *E. nicaeensis* roots were packed into a Soxhlet extraction flask and 250 g of 96 % ethanol was added for continuous extraction with heating for 2 h, and then left overnight in the solvent. After removal of the solvent by evaporation on a rotary vacuum evaporator, the obtained extract (25 g) was fractionated by DCFC using silica gel as the stationary phase and a mixture of petroleum ether and acetone in varying volume ratios as the mobile phase. Fraction F3 (2.1 g), obtained by elution with 15 % acetone in petroleum ether, was further fractionated by DCFC on silica gel using a mixture of petroleum ether and acetone in different ratios (98:2, 97.5:2.5, 95:5, 90:10, 80:20) as the mobile phase. The collected fractions were monitored by TLC, and similar fractions were combined, resulting in thirteen fractions (1–13). Fraction F3/10 (367.5 mg) was subjected to separation by NP-HPLC on silica gel (Zorbax Rx-SIL column, 250 mm $\times$ 9.4 mm, 5 μm) with *n*-hexane and acetone (95:5, isocratic mode, flow 3 mL/min, 25 °C, 227 nm, stop time 30 min, post time 1 min), yielding six subfractions, F3/10/I to F3/10/VI. The final separation of fraction F3/10/VI was carried out on RP-HPLC using a Zorbax XDB C18 column (250 mm $\times$ 9.4 mm, 5 μm) and a mixture of water and acetonitrile (ACN) in gradient mode as the mobile phase (50–80 % ACN (0–10 min), 80–90 % ACN (10–15 min), 90–100 % ACN (15–21 min), flow 4 mL/min, 25 °C, 227 nm, stop time 21 min, post time 2 min). As a result of this purification, lumiphorbol (0.7 mg) was isolated. The NMR spectra and HRMS data are available in Supplementary material to this paper.

RESULTS AND DISCUSSION

Luminicaenin A (**1**) was isolated as a colourless amorphous substance. The ion $[\text{M}+\text{H}]^+$ at m/z 479.2424 in HRESIMS indicated the molecular formula $\text{C}_{29}\text{H}_{34}\text{O}_6$, (Calcd. for $\text{C}_{29}\text{H}_{35}\text{O}_6$, 479.2428), corresponding to 13 degrees of

unsaturation. NMR spectra (Table I) revealed the functional groups, such as a 3-keto group (δ_C 218.1 ppm), a tertiary OH (δ_H *ca.* 5.2 ppm, *brs*; δ_C 82.6 ppm, C-9), and two ester residues, 12-benzoate (δ_H 8.03 ppm, *d*, 8, 2H, δ_H 7.46 ppm, *t*, 8 2H and δ_H 7.58 ppm, *t*, 1H; δ_C 166.2 ppm and δ_C 130.3, 129.9, 128.6 and 133.3 ppm) and 13-acetate (δ_H 2.12 ppm, *s*, 3H; δ_C 173.0 and 21.2 ppm). When the saturations originating from the mentioned functional groups are subtracted from the total number of saturations, six more unsaturations remain in the skeleton, which must be distributed through six rings given that in the NMR spectra there are no signals originating from carbon-carbon double bonds. The clue for a cage structure containing six rings was mostly obtained from HMBC, COSY and NOESY correlations shown in Fig. 1, as well as by the comparison of 1H -NMR spectral data with those of the related lumiphorbols obtained by the intramolecular [2+2] photocycloaddition of 4 α -phorbol derivatives.¹⁷

TABLE I. 1H - and ^{13}C -NMR data of **1** (500 MHz for 1H , and 125 MHz for ^{13}C , $CDCl_3$, TMS)

Compound	1H (δ / ppm, <i>J</i> / Hz)	^{13}C (δ / ppm)
1	2.30, <i>t</i> , 6	43.4
2	—	42.9
3	—	218.1
4	2.43, <i>brs</i>	46.48
5α	3.24, <i>d</i> , 12	38.7
5β	1.59, <i>brd</i> , 13	—
6	—	46.50
7	2.75, <i>t</i> , 5	52.2
8	1.76, <i>dd</i> , 10, 5	55.0
9	—	82.6
10	2.72, <i>t</i> , 5	44.2
11	1.67, <i>qui</i> , 7	51.5
12	5.78, <i>d</i> , 8	81.2
13	—	68.2
14	1.49, <i>d</i> , 10	30.3
15	—	28.5
16	1.20, <i>s</i>	24.1
17	1.39, <i>s</i>	18.0
18	1.07, <i>d</i>	14.4
19	1.12, <i>s</i>	7.2
20	1.02, <i>s</i>	23.3
12-OR	—	—
1'	—	166.2
2'	8.03, <i>d</i> , 8	130.3
3'	7.46, <i>t</i> , 8	129.9
4'	7.58, <i>t</i> , 8	128.6
5'	—	133.3
13-OR	—	—
1''	—	173.0
2''	2.12, <i>s</i>	21.2

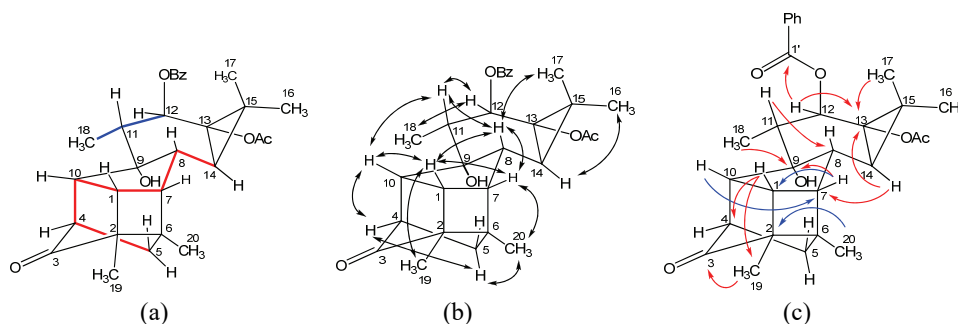


Fig. 1. 2D correlations of **1**; a) COSY correlations; spin system A (red), spin system B (blue), b) NOESY correlations and c) HMBC (H→C) correlations (the correlations occurring across four-membered ring marked blue).

The COSY spectrum of **1** has shown two independent spin systems A and B. The first one (A) included H-1, H-4, H-5, H-7, H-8, H-10 and H-14, while the second (B) included protons H-11, H-12 and H-18. The COSY correlation H-1/H-7 indicated connectivity C(1)–C(7), which is also supported by the HMBC correlation H-7/C-1. At the same time, the HMBC correlations H3-20/C-2 and H-10/C-7 (Fig. 1c, marked blue) were in accordance with the C(1)–C(7) connectivity in the four-membered ring. The NOEs (Fig. 1b) also supported the proposed cage structure of **1**. The structure of **1** was interpreted as 4,20-deoxy-4 α -lumiphorbol-12-benzoate-13-acetate.

Unlike the tricyclic core, which is commonly found in Euphorbiaceae, lumiphorbol derivatives are extremely rare. To the best of our knowledge, only one diterpene with the lumiphorbol structure, *i.e.*, 12-*O*-palmitoyl-4-deoxy-16-hydroxylumiphorbol-13-acetate isolated from *Aleuritis fordii* (Euphorbiaceae) from Japan has been described in the literature so far.²⁰ Otherwise, lumiphorbol derivatives reported in the literature are the products of the intramolecular [2+2] photocycloaddition reaction of 4 α -phorbols carried out by irradiating with UV light ($\lambda = 254$ nm).^{16,17}

CONCLUSION

This study identified the roots of *E. nicaeensis* as a source of an extremely rare lumiphorbol diterpene skeleton. Considering all the facts presented, it can be concluded that lumiphorbols represent a significant class of compounds, with unique structural features and still unexplored biological activities due to their rarity, which indicates the need for further chemical and pharmacological research.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13212>, or from the corresponding author on request.

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ИЗВОД

ПРВИ ДОКАЗ ЛУМИФОРБОЛА КАО МЕТАБОЛИТА ВРСТЕ РОДА *Euphorbia*

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Euphorbia nicaeensis All. је цветница из породице Euphorbiaceae. Наше претходно истраживање је показало да је латекс ове врсте богат извор јатрофанских дитерпена. Због изузетних биолошких активности које ови дитерпени показују, истраживање је проширено на корен врсте у потрази за новим дитерпенима. Из корена *E. nicaeensis*, прикупљеног у Делиблатској пешчари, изолован је по први пут из врста рода *Euphorbia* дитерпен који припада изузетно реткој класи лумифорбола. Структура изолованог једињења одређена је применом спектроскопских техника (1D- и 2D-NMR), као и масене спектрометрије високог разлагања (HRESIMS).

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